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CHARACTERIZATION OF ANTIOXIDANT ACTIVITY OF PEPTIDE FRACTIONS FROM CHINESE GIANT SALAMANDER (*Andriaus davidianus*) PROTEIN HYDROLYSATE

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In this current work the muscle of Chinese giant salamander was hydrolysed by trypsin for seven hours to get protein hydrolysates which were purify by superdex peptide 10/300 GL and Reverse phase chromatography (PR-HPLC) for their identification of antioxidant activity. The degree of hydrolysis was determined (88.87 % protein content). Five different concentrations of protein hydrolyzed samples, AKTA fraction and seven RP-HPLC fractions were analyzed (F1, F2, F3, F4, F5, F6 and F7). Antioxidant activity of this samples were determine by four difference assay namely DPPH, β-carotene/linoleic acid, reducing power and metal chelating. The result of RP-HPLC fractions shows higher antioxidant activity in all assay which have strong correlation with concentration, the DPPH inhibition were 68.39 %, 57.96 % and 49.15 % for F7, F3 and F1 respectively; β-carotene activity were F7 (98.21 %), F5 (83.50 %) and F6 (79.00 %); reducing power F4 (0.89), F3 (0.67) and F2 (0.51) at 700 nm and metal chelating F7 (97.29), F5 (85.50) and F6 (83.00). The results also concluded that F7 was more active in radical scavenging while lowest value for reducing power assay.
Keywords: Chinese giant salamander, antioxidant activity, peptide fractions, AKTA superdex peptide 10/300 GL

INTRODUCTION

Chinese giant salamander (*Andrias davidianus*) is the largest salamander in the world, and fully aquatic with many adaptations for lifestyle. It grows up to 1.8 metres in length, though most individuals found today are considerable smaller Liu *et al*. (2010). Numerous studies have proved that the muscle of Chinese giant salamander has high nutritional value and is rich in quality protein, plentiful essential amino acid and delicious amino acid at higher content levels Li *et al*., (2012). The fatty acid composition of Chinese giant salamander consists of higher percent of unsaturated fatty acid, especially DHA Liu *et al*., (2010). The current situation of research of Chinese giant salamander meat based foods is analyzed and the development of heath care food such as protein and amino acids products and function fates is predicted to be the main direction of exploitation of Chinese giant salamander meat based food Liu *et al*., (2010.

In this study, the Chinese giant salamander as raw materials was evaluated for its antioxidant properties through its protein hydrolysate and peptides fractions. Currently, the study on antioxidant from natural source becomes increasing in scientific research due to emerging disease that result from artificial antioxidants. For example antioxidant activities of peptide fractions from capelin protein hydrolysates (Amarowicz and Shahidi, 1997) essential oil Amorati *et al*., (2013) salmon fraction Borawska *et al*., (2016) tilapia Foh *et al*., (2010) Mediterranean fish species García-Moreno *et al*., (2014) and Chinese giant salamander skin secretion glycopeptides Li *et al*. (2012).

Antioxidants are molecular species that inhibits the oxidation of other molecule that can produce free radicals leading to chain reactions and may damage cells. This approach is done by chemistry of several test developed to assay the antioxidant activity of natural extract which based on the reaction of the potential antioxidant with some colored persistent radical (e.g., DPPH) or with some oxidizing none radical species for example (reduction of Fe^{3+} to Fe^{2+}), β-carotene, iron chelating , reducing power and others Amorati *et al*. (2013).

Many chronic diseases like atherosclerosis, carcinogenesis, diabetes, coronary heart and cardiovascular diseases, are associated with oxidative stress and imbalance between oxidizing species and natural antioxidants in the body Segura Campos *et al*., (2010). This process result to a lowering of essential quality of food and reduce food shelf life especially quality of lipid containing foods during processing and long time storage Gao *et al*., (2010). To overcome the consequence of oxidative stress, different methods are used in food industry and its related field in order to prevent or reduce to certain extant the oxidative damage of lipids. For the past decade now strong chemicals with antioxidant activity are used as food additives to suppress the oxidative stress caused by radicals, chemical like butylated hydroxyanisole, butylated hydroxytoluene and *n*-propyl gallate were used as antioxidant

activity against several oxidation form Segura Campos *et al*., (2010). However, many research reporting on the health risk of using this chemical as the only way to strengthening the antioxidant defense system since it cause potentially *deleterious* to human health Powqnall *et al*., (2010).

It has been proven that fish protein hydrolysates could demonstrate biological activities by the action of bioactive peptides, which allows classifying them as essential ingredients of functional foods and nutraceuticals. Therefore, exploring and developing safe and non-toxic natural antioxidants has been a popular topic in recent decades. To the best of our knowledge no study that work on antioxidant activity from protein hydrolysate and peptide fractions extracted from Chinese giant salamander, this study therefore, aimed to determine the antioxidant activity of protein hydrolysate and its fractionated peptide obtained by AKTA superdex peptide 10/300 GL and Reverse phase chromatography (RP-HPLC).

MATERIAL AND METHODS

Trypsin (EC 3.4.21.4) from porcine pancreas was purchased from Sinopharm chemical reagent Co., Ltd was used to prepared protein hydrolyses with deference conditions. Male healthy Chinese giant salamanders (*Andrias davidianus*) with three or four year old with body length of 70–100 cm and weight of about 4 kg was provided by Yangzhou Rongda Agricultural Science and Technology Co., Ltd (Jiangsu, China). 1, 1-diphenyl-2-picrylhydrazyl (DPPH), β-carotene, Linoleic acid Ascorbic acid and Ferrozine are purchased from Sigma-Aldrich Co. Ltd. All other reagent and chemical were of analytical grade

***Preparation of protein hydrolysates (PHs*)**: The protein hydrolysate was prepared as the method describe by Adler Nissen (1979). with slight modifications. Before addition of enzyme 0.06 enzyme substrate ratio (E/S), slurry was homogenized and adjusting to the desired pH (7.0) and temperature (60˚C) which was kept constant for all hydrolysis time. The reaction was conducted for six hours and after every one hour of hydrolysis time aliquots of the samples were drawn from reaction mixture for the determination of degree of hydrolysis. To terminate the reaction of enzyme, the solution was heated to 80 ˚C to inactivate the enzyme. The hydrolysates were centrifuged at a speed of 10,000 rpm at 4 ˚C for 15 minutes and supernatants were dialyzed and freeze dried stored at -20 ˚C until further analysis.

***Determination of degree of hydrolysis (DH*)**: Degree of hydrolysis was calculated by formal titration method following the method of (Taylor 1957) with slight modifications, 10 mL of Protein Hydrolysates (PH) sample was added with an equal amount of distilled water, than the mixture was adjusted to pH 7.5 using 0.1 M NaOH. Then 10 mL of 38 % (v/v) formaldehyde solution was added into the mixture and left for 5 minutes at room temperature and titration was continued to the end point at pH 9 with 0.1 M standard NaOH solutions and the volume consumed was used to calculate the amount of free amino group. DH was determined by dividing the amount of free amino group by total amount of nitrogen by using equation 1.

$$\text{Degree of hydrolysis (\%)} = \frac{\text{\% free amino groups}}{\text{\% nitrogen content}} \times 100 \qquad (1)$$

Purification of protein hydrolysate by size-exclusion chromatography and Reverse phase chromatography (RP-HPLC): Powdered hydrolysates was fractioned by size-exclusion chromatography (AKTA gel chromatography) with column superdex peptide 10/300 GL, sample was eluted by distilled water at flow rate of 1mg/min at 220 nm absorbance, three fractions were collected and noted as ATKA fraction AKTA 1, AKTA 2 and AKTA 3 than lyophilized and used to detecting antioxidant activity. Among fractions only one fraction AKTA 2 shows highest activity and used for purifications.

The fraction showing high antioxidant activity in size-exclusion chromatography was further purified by an analytical Reverse phase-HPLC (RP-HPLC) with a C18 column XselectTMCSH130 (4.6×250 cm) at a flow rate of 1 mL/min with a linear gradient of 0.1 % acetonitrile containing 0.065 % TFA to 20 % acetonitrile containing 0.05 % TFA in 35 min, 20 % acetonitrile (0.05 % TFA) to 80 % acetonitrile (0.05 % TFA) in 10 min, 80 % acetonitrile (0.05 % TFA) to 2 % acetonitrile (0.065 % TFA) in 10 min sequentially. The elution peaks were detected at 280 nm. Seven peptide fractions with different retention time were collected and noted as fraction FI, F2 F3 F4 F5 F6 and F7, lyophilized and stored in -20 for further evaluation of antioxidant activity.

***Determination of antioxidant activity*:** The DPPH radical-scavenging activity of the hydrolysates was determined as described by (Jemil, 2014) with slight modifications. An aliquot of 250 µL protein hydrolysate or fractions was mixed with 500 µL Tris buffer solution (50 mM, pH 7.4) and with 4250 µL of a daily-prepared solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) at 0.1 mM in methanol. The mixture was then kept at room temperature in the dark for 30 min, and the reduction of DPPH radical was measured at 515 nm. A blank was prepared in the same way by using distilled water instead of sample, and sample control was also made for each sample by adding methanol instead of DPPH solution. Then, DPPH radical scavenging activity was calculated according to the equation 2.

$$\text{DPPH scavengig activity \%} = \left(1 - \frac{A_{sample} - A_{control}}{A_{blank}}\right) \qquad (2)$$

***Reducing power assay* :**The assay was determined according to the method of (Jemil, 2014) 1 mL of fractions and protein hydrolysate (0.1-1 mg/mL) concentrations was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) following 2.5 mL of 1% (w/v) potassium ferricyanide solution. After 30 min at 50 ˚C of incubation, 2.5 mL of 10 % (w/v) TCA was added and the reaction mixtures centrifuged at 10,000×g for 10 min. Finally, 2.5 mL of the supernatant solution from each sample

mixture was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% (w/v) ferric chloride. After 10 min reaction time, the absorbance of the resulting solutions was measured at 700 nm. Increased absorbance of the reaction mixture means increased reducing power. The control was prepared in the same manner, except that distilled water was used instead of sample.

***Antioxidant assay using the β-carotene bleaching method*:** This method was determined as described by (Jamil, 2014) with slight changes. A stock solution of β-carotene/linoleic acid was prepared by dissolving 0.5 mg of β-carotene, 25 µL of linoleic acid and 200µL of Tween 40 in 1 mL of chloroform. The chloroform evaporated under vacuum in a rotator evaporator at 40˚C; then 100 mL of distilled water was added and the resulting mixture vigorously stirred. Aliquots (2.5 mL) of the β-carotene/linoleic acid emulsion were transferred to test tubes containing 0.5 mL of each protein hydrolysate or fractions at different concentrations. Following incubation for 2 h at 50 ˚C, the absorbance was measured at 470 nm. The control tube contained no sample. The antioxidant activity was evaluated in terms of bleaching of β-carotene using the equation 3.

$$\mathrm{AA}(\%) = \left[1 - \frac{A_O - A_t}{A'_O - A't}\right] \times 100 \qquad (3)$$

Where A_0 and A'_0 are the absorbencies of the test sample and the control, respectively, measured at time zero; and A_t and A'_t are the absorbencies of the sample and the control, respectively, measured after incubation for 120 min.

***Iron (Fe^{2+}) chelating activity assay*:** The iron chelating activity of the protein hydrolysates or fractions was prepared following the method described by Morales-Medina [17] with slight modifications. 1 mL of protein hydrolysate solution or protein fractions (0.1-1 mg/mL) was mixed with 3.7 mL of distilled water and 0.1 mL of ferrous chloride 2 mM. After 3 min, by adding 0.2 ml of ferrozine 5 mM the reaction was inhibited. Then, sample was stirred and incubated at room temperature for 10 min. The absorbance was measured at 562 nm. As blank water was used instead of sample and as for the sample control ferrozine was not added. The chelating activity was calculated with Equation 4.

$$\text{Metal chelating activity}, \% = \left(1 - \frac{\mathrm{A_{sample} - A_{control}}}{\mathrm{A_{blank}}}\right) (4)$$

***Statistical Analysis*:** The data were obtained in triplicate and were reported as mean ± standard deviations. Analysis of variance was carried out by SPSS program (version 19.0) at $p < 0.05$.

RESULTS AND DISCUSSION

***Degree of hydrolysis*:** Degree of hydrolysis (DH) is defined as the percentage of free amino groups cleaved from protein, which calculated from ratio of α-amino nitrogen (AN) and total nitrogen (TN) (Taylor 1957). Enzymatic hydrolysis of Chinese giant salamander has been shown to produce peptides with different bioactive properties. The protein muscles with proximate protein composition of 88.87±0.11 % were hydrolysed for six hours and produce comparable value of DH. The result show that there are significance increasing of DH with increasing of time, Fig. 1 show change in degree of hydrolysis which range from 17.38 % to 24.251 % at 0.06 (E/S).

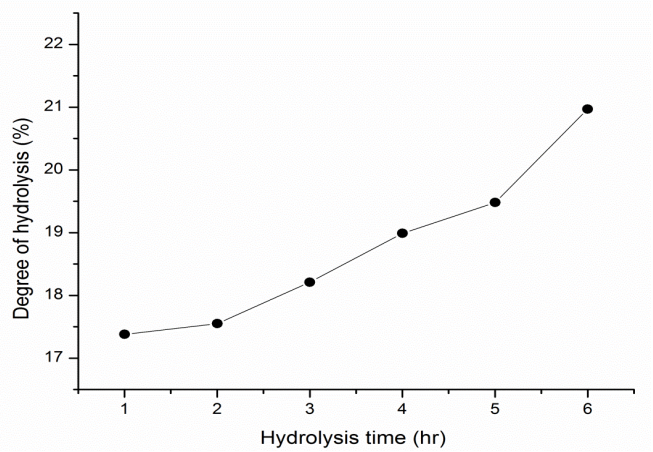

Figure 1: Degrees of hydrolysis (DH) of Trypsin 0.06 (E/S) produced for six hours

DPPH radical scavenging: The scavenging capacity of protein hydrolysate or peptide fractions obtained from Chinese giant salamander was measured against 1,1-Diphenyl-2-picylhydazyl DPPH ($C_{18}H_{12}N_5O_6$), this was took place by an old electron of nitrogen atom in DPPH being reduced by receiving a hydrogen atom from antioxidant to the corresponding hydrazine [18, 19]. The reaction give rise to violate color with an absorption in methanol around 517 nm, the color was lost as the protein hydrolysate or peptide fractions donate their hydrogen atoms and DPPH reduced its electron. The percentage inhibition seems to increase as the concentration of protein hydrolysate or peptide fractions ar1616increased from 0.1 to 1 mg/mL as shown from Figure 2. The protein hydrolysate had percentage inhibition range from 16.17 to 54.73 %, the smaller concentration result lower inhibition property. Compared to peptide fractions where the inhibition range from 22.03 to 56.75 %, also there was an improvement observed in inhibition property after the protein was fractionated as shown in Figure 5. Meanwhile the inhibition of RP-HPLC fractions was seem to be higher compared to both protein hydrolysate and AKT fractions. Elution fractions F7, F1 and F3 were 68.39 %, 57.96% and 49.15% respectively were the dominate peaks that show best indicator for antioxidant activity and show distinct inhibition properties. In completion radical scavenging activity of protein hydrolysate and its peptide fractions was varies considerably, in which protein hydrolysate sample was seem to have lower antioxidant activity then its corresponding peptides.

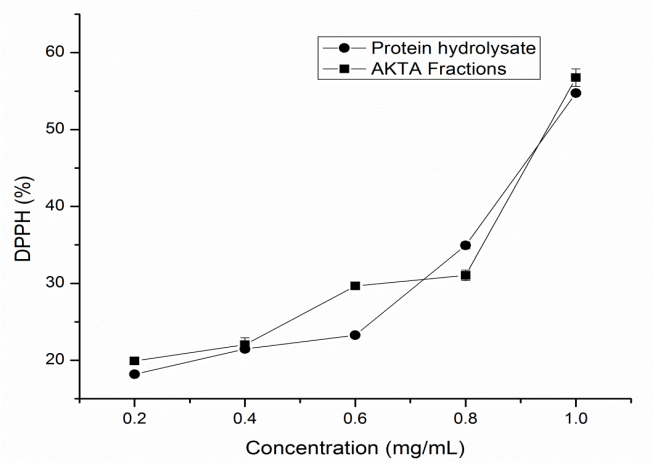

Figure 2: Antioxidant activity of DPPH assay of Chinese giant salamander protein hydrolysate and peptide fraction from AKTA superdex peptide 10/300 GL

Reducing power assay: The assay of reducing activity was based on the reduction of ferricyanide (Fe^{3+}) complex to the ferrous (Fe^{2+}) form in the presence of antioxidant in the samples Liu *et al*., (2013). Figure 3 show the reducing power of protein hydrolysate, and peptide fraction from AKTA while the reducing power for the fractions obtained by RP-HPLC reported in Figure 4. Peptide fractions obtained from RP-HPLC was record to have higher absorbance compared to all samples and in this assay increasing in absorbance reflect higher reducing power and therefore that peptide fractions originate from this assay have higher reducing power Pownall *et al*., (2010). Fraction F4 have highest reducing power 0.89±0.03 (Abs 700 nm) followed by F3 having the absorbance of 0.67±0.06, these were the comparative result obtained from protein hydrolysate of grass cap Liu *et al*., (2013). and protein fraction of cod Fervin *et al*., (2014). In fact the peptide fractions obtained from RP-HPLC have higher reducing power compared to protein hydrolysates which have lowest absorbance. Protein hydrolysate shows the lowest absorbance four times than that obtained from fractionation 0.14 ±0.01. Along the same line, Borawska *et al.* demonstrate that salmon protein fraction shows higher reducing power more than in natural condition of hydrolysate.

***β-carotene bleaching activity*:** The β-carotene activity for the protein hydrolysates and AKTA fractions are reported in Table 1 and that of RP-HPLC are reported in Figure 5. Amino acid especially hydrophobic tend to have strong antioxidant activity against lipid derived radical due to interaction between hydrophobic amino acid and lipids Liu *et al*., (2013). β-carotene undergoes rapid discoloration in the absence of an antioxidant; this is due to the coupled oxidation of β-carotene and linoleic acid, which generates free radicals (Jamil 2014).

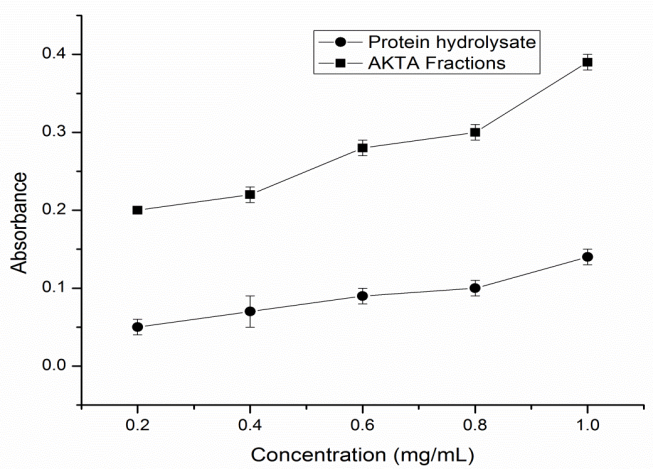

Figure 3: Reducing power activity of protein hydrolysate from Chinese giant salamander and peptide fraction from AKTA superdex peptide 10/300 GL

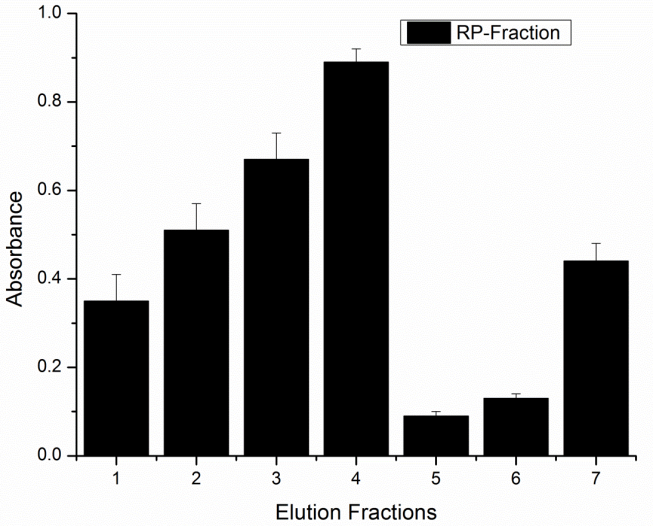

Figure 4: Reducing power activities of seven collected fractions from RP-HPLC

In this case, fraction from RP-HPLC and protein hydrolysate exhibited strong β carotene activity over two hours. RP-HPLC fractions were the most active and the activity range from 77.5 ±1.31 % to 98.21 ±1.90 % at different concentrations. The fraction F7 with concentration of 2.869 mg/mL show highest β-carotene bleaching inhibition 98.21 ±1.90 %, fraction F5 marked as second fraction with 83.50 ±1.50 % inhibition at the concentration of 0.176 mg/mL this was followed by protein hydrolysate samples, which was range from 29.77±0.01 % to 81.35±0.15 % at (0.1 to 1.0 mg/mL). The AKTA fractions had weakest activity and were observed to range from 4.75±0.35 (%) to 39.95±0.64 (%) at concentrations range from 0.1 to 1.0 mg/mL as show in Table 1. This trend are reported by protein hydrolysate of fish

meat fermented by *Bacillus subtilis* that all protein hydrolysates exhibited the oxidation of β-carotene [16].

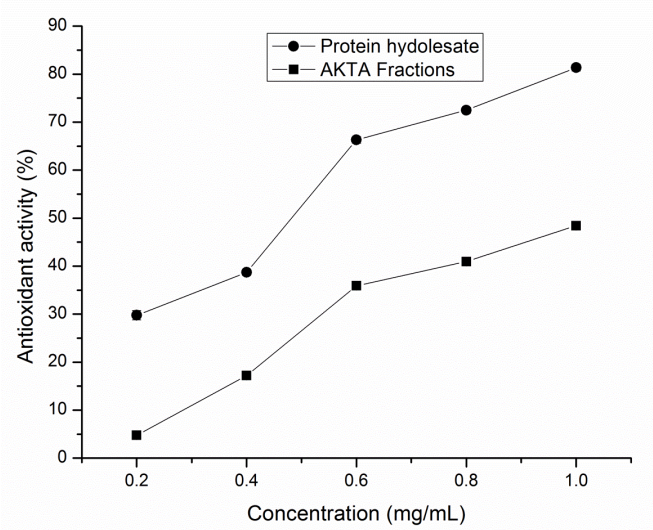

Figure 5: β-carotene activity of protein hydrolysate from Chinese giant salamander and peptide fraction from AKTA superdex peptide 10/300 GL

***Iron (Fe^{2+}) chelating activity*:** The results of chelating effect of protein hydrolysate and peptide fraction are reported in Table 1 for protein hydrolysate and AKTA fraction and Figure 6 for RP-HPLC fractions. Metal chelating involves aliphatic compounds, where a five-member ring is formed, which is composed of the metal ion and two chelating ligands Pownal *et al*., (2014) , the chelating activities of the samples increase with the increasing of the concentration. The most powerful activity was observed from peptides fractions that were collected by RP-HPLC, and the chelating effect was range from 77.5 ±1.31 (%) to 97.29±1.90 (%). While fraction F7 have the highest metal chelating activity 97.29 ±1.90 (%) the remaining fractions composed considerable similar range of activity. The fraction from AKTA is the second in activity compared to fraction from RP-HPLC which has the chelating activities range from 52.05 ±1.41 (%) to 67.10 ±4.57 (%) in which as concentration increase and the activity increase. Protein hydrolysate show the lowest chelating activity which range from 10.20±3.52 (%) to 30.17±1.12 (%).In comparison many research clamming that peptide fractions composed huge metal chelating activity than protein hydrolysate. Peptide fractions derived from cottonseed protein hydrolysate [12], peptide fraction from tuna dark muscle [19], fractionation of whey protein [21] However protein hydrolysate could have more metal chelating activity depend on enzyme type and time length of hydrolysis time Pownal *et al*.,(2014).

Table 1: Metal Chelating activity and β-carotene activity of protein hydrolysate and AKTA fraction

Concen-tration (mg/mL)	Metal Chelating activity (%)		Beta carotene (AA %)	
	Protein hydrolysate	AKTA Fractions	Protein Hydrolysate	AKTA Fractions
0.1	10.20±3.52	52.05±1.41	81.35±1.12	4.75±0.35
0.2	13.20±0.54	54.76±0.14	72.48±0.69	11.65±0.54
0.4	16.83±0.21	59.12±0.87	66.29±0.66	17.20±0.42
0.6	20.75±0.32	60.87±1.76	38.69±0.59	28.54±0.12
0.8	28.30±0.31	64.55±0.79	39.19±0.57	35.09±0.28
1.0	30.17±1.12	67.10±4.57	29.77±0.98	39.95±0.64

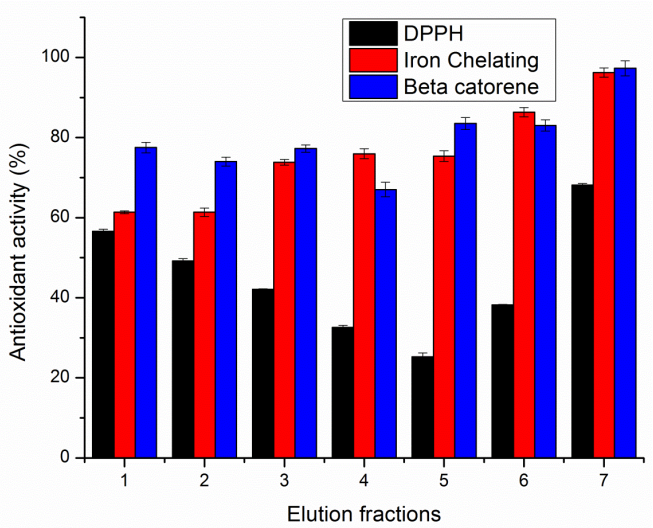

Figure 6: D PPH, Metal chelating activity and Beta carotene of RP- HPLC fractions (F1-F7)

Conclusion: This study demonstrated that, there is in vitro antioxidant activity of protein hydrolysate and peptide fractions obtained from Chinese giant salamander by ATKA superdex peptide and RP-HPLC. The result reveals that purified fractions show highest activity in DPPH, B-carotene and iron chelating also show lowest reducing power. The next step of this study is to isolate and identify their amino acid sequence of the most important natural compound from these bioactive fractions which will give better comprehensive of peptide structure and functionally relation and conduct in vivo antioxidant assay to validate the result obtained in animal model .Moreover natural antioxidant obtained will be helpful for industrial processing to prolong shelf life and stability of the food also to protect human health by using as fortify material or as food additive to delay the deterioration of food due to oxidation

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